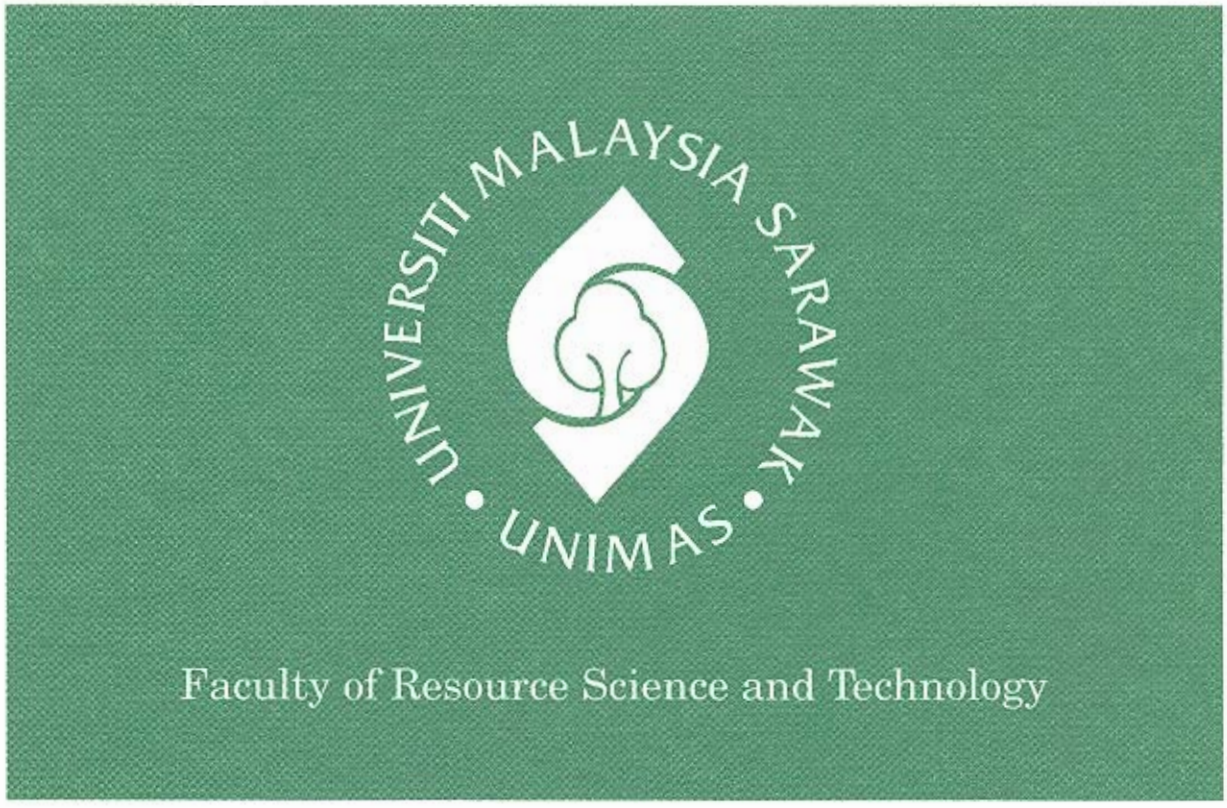

CRYOPRESERVATION OF *ARTOCARPUS ODORATISSIMUS* BLANCO SEEDS USING DEHYDRATION TECHNIQUE

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THIS PROJECT IS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE WITH HONOUR (RESOURCE BIOTECHNOLOGY)

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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ABSTRACT

Cryopreservation is regarded to be the most suitable method for establishment of a long term conservation of plant germplasms especially for seed which are recalcitrant. Among methods of cryopreservation, dehydration has been frequently used since it is simple, economical, and gives high survival and regrowth rates for various plants. This study was aimed at assessing the suitability of dehydration techniques in preservation of *Artocarpus odoratissimus* seeds. Dehydration using silica gel for 24 hours gave the highest germination which was 88.0%, and the moisture content was 34.80%. The highest germinations using sucrose at 0.2 M for 60 minutes and laminar air flow for 8 hours were both 86.0% with moisture content of 42.82% and 40.11% respectively. Regression analysis predicted that *A. odoratissimus* seeds could be stored for 3 months and 4 days if treated using silica gel. When the seeds were treated using laminar air flow, it could be stored in the liquid nitrogen for 4 months and 8 days. While, when the seeds were treated with sucrose solution, these seeds could be stored for 4 months and 5 days.

Key words: Cryopreservation, dehydration, *Artocarpus odoratissimus*, moisture content, germination

ABSTRAK

Krioawetan merupakan kaedah yang paling sesuai untuk pemeliharaan jangka masa panjang bagi tumbuhan terutama untuk biji yang mempunyai jangka hayat yang pendek. Antara teknik krioawetan yang kerap digunakan adalah penghidratan kerana kaedah ini lebih mudah, murah dan memberikan kadar kebolehidupan dan pertumbuhan semula yang tinggi untuk pelbagai tumbuhan. Kajian ini dilakukan untuk menilai kesesuaian teknik penghidratan dalam penyimpanan biji Artocarpus odoratissimus. Penghidratan menggunakan silika gel selama 24 jam memberikan peratus percambahan yang tertinggi iaitu 88.0% dengan peratus kandungan lembapan 34.80%. Manakala bagi teknik larutan sukrosa (0.2 M, 60 minit) dan laminar air flow (8 jam) masing-masing menunjukkan percambahan pada kadar 86.0%. Peratus kandungan lembapan pula adalah 42.82% dan 40.11% untuk kedua-duanya. Analisis regresi menunjukkan biji A. odoratissimus dapat disimpan selama 3 bulan 4 hari apabila dihidrat menggunakan silika gel. Biji yang dihidrat menggunakan teknik laminar air flow dapat disimpan dalam cecair nitrogen selama 4 bulan 8 hari. Manakala, biji yang dihidrat menggunakan larutan sukrosa dapat bertahan selama 4 bulan 5 hari.

Kata kunci: Krioawetan, penghidratan, Artocarpus odoratissimus, kandungan lembapan, percambahan

CHAPTER 1

INTRODUCTION

Artocarpus odoratissimus or well known as 'tarap', is a fruit which belongs to the genus Artocarpus. The name Artocarpus is derived from the Greek words 'artos' which means bread and 'karpos' refers to fruit. This name was established by Johann Reinhold Forster and J. Georg Adam Forster, the father and son team of Botanist aboard the HMS Resolution on James Cook's second voyage (Wikipedia, 2004). Artocarpus is a genus of about 60 species of Southeast Asian origin and the Pacific and it is belonging to the mulberry family Moraceae. According to Wikipedia (2004), Artocarpus is closely related to and rather difficult to distinguish from the genus Ficus. Tarap and *Artocarpus champeden* (cempedak) are the most well known species of the genus.

Wikipedia (2004) also stated that the species in this genus are all laticiferous trees and a few shrubs, of which the leaves, the twigs and the stem can produce a milky sap. The trees are monoecious, with unisexual flowers, with both sexes on the same plant. The small, greenish, female flowers grow on short, fleshy spikes. After pollination they grow into a syncarpous fruit, which can become very large. The ovary is superior. Stipulated leaves vary from small and entire (*Artocarpus integer*) to large and lobed (*Artocarpus odoratissimus*) (Wikipedia, 2004).

A. odoratissimus is found extensively in Sabah, Sarawak and Brunei Darussalam. It can be found in secondary as well as primary forests. It is also widely cultivated in the villages especially in the Miri and Limbang Divisions where excellent varieties with large fruits are

found. It is an evergreen and sometimes with low buttresses tree which diameter and height can reach 40 cm and 25 m, respectively (CPCP, 2003).

The fruit is globular to oblong in shape and has a long, stout fruit stalk. A large fruit is about the size of a breadfruit and is covered with soft, fleshy spines (Voon *et al.*, 1992). When ripe, the fruit has a greenish-yellow colour, becomes soft and emits a powerful odour. At this stage, the skin is easily pulled open to reveal many small segments of glistening while flesh each with a small seed. The flesh is very aromatic, very sweet and soft. Voon *et al.* (1992) also stated that the seeds of *A. odoratissimus* are a favourite titbit when fried or roasted with a pinch of salt. Ripening fruits must be plucked from the tree as it will drop when beginning to rot.

The large fruit is esteemed for the sweet, juicy, aromatic perianths surrounding the seeds, which can be eaten fresh or used as an ingredient in cakes. The fruit is said to have a finer and more delicate flavour than the jackfruit. It has a delicious nutty flavour. Young fruits are also cooked in coconut milk and eaten as a curried vegetable.

According to Towill (1991), cryopreservation refers to placing and holding of biological materials at low temperature in a manner such that viability is retained after thawing. Usually, liquid nitrogen is used to store the living organism at ultra-low temperature. This long term conservation method is increasingly used in the management of crop plant genetic resources and it is also an important component of many plant biotechnology programmes. Cryopreservation apply for storage of a wide variety of plant germplasm such as buds, seed, seed parts, twigs, tissue culture, cells and DNA. Besides, it is also used in preserving algae,

bacteria, gametes, red blood cells, fish and frog spermatozoa, virus and many other germplasms.

Currently, four types of cryopreservation are dehydration, encapsulation, slow freezing and vitrification had been applied to various kinds of living organisms. Dehydration technique had been intensively studied to preserve various kinds of seeds. This technique consists of three different ways which are laminar air flow, silica gel and sucrose solution dehydrations.

1.0 Problem Statement

A. odoratissimus originated from South East Asia forests. Due to rapid development, rampant and unlicensed timber activities, the *A. odoratissimus* in these countries, could face extinction. So, cryopreservation has a great potential as a way to preserve the germplasm. Through this technique, *A. odoratissimus* seed could be preserve as germplasm, with the original characteristics and store for a long period without serious damage.

Although various plant species had been preserved by cryopreservation, no specific effort had been done on *A. odoratissimus* seeds. Long term storage of recalcitrant seeds by conventional methods has by far been unsuccessful. In this study, *A. odoratissimus* seeds, a recalcitrant species had been attempted at for long term preservation using cryogenic means, particularly dehydration technique.

1.1 Objectives

The objectives of this study are as follows:

1. To assess the suitability of cryopreservation in the conservation of *A. odoratissimus* seeds.
2. To evaluate the effectiveness of dehydration techniques of cryopreservation in preservation of *A. odoratissimus* seeds.
3. To estimate the longevity of *A. odoratissimus* seeds kept or stored in liquid nitrogen.

CHAPTER 2

LITERATURE REVIEW

2.1 Seed Characteristics

CPCP (2003) reported that in a series of fruit taken from wild and cultivated trees, fresh weight per seed ranged from less than 0.5 g to more than 1.0 g with the moisture content of the seed was 38 - 44%. Mean number of seeds per fruit varied from 50 - 147. While on dry basis, the seed contains 11 - 15% protein, 20% fat and 54 - 72% carbohydrates.

The seeds are many, whitish, 8 x 15 mm in size, smooth surface and readily separated from the flesh (CPCP, 2003). The pattern of germination for this species is through hypogeal. During hypogeal germination, cotyledons or comparable storage organs remain beneath the soil while the plumule pushes upward and emerges above the ground (Copeland and McDonald, 1995). Epicotyl is the rapidly elongating structure in this type of germination.

2.1.1 Category of Seeds

Seeds can be divided into two categories according to their natural characteristics. This can be seen from its way of survival, moisture content and its sensitivity during storage. It is crucial for the survival of animals and plants to keep a high, stable level of water in the organisms. Though some seeds seemed very dry when they are collected, the water content, usually called moisture content, of seed is a very important factor to consider in relation to storability and survival of the seed.

Many seeds can be dried to very low moisture contents and may be stored that way for years, they are called orthodox seeds. Other seeds, often tree seeds from the humid tropics, cannot be dried, or only slightly dried. They are very difficult to handle and store and are therefore called recalcitrant seeds, as in the case of *A. odoratissimus* seeds.

Recalcitrant seeds generally store best at 15°C and with moisture content few percents below the moisture content of these seeds when they were collected. Low moisture contents and low temperatures damage the seeds (Thomsen and Stubsgaard, 1998). Besides having high moisture content, recalcitrant seeds are also difficult to store due to large seed size.

Thomsen and Stubsgaard (1998) also reported that, for true recalcitrant species minimum moisture contents will often be around 35 - 40%. The 'intermediate' species (between orthodox and recalcitrant) usually can be dried down to 10 - 20% moisture content.

Even at optimal conditions for seeds of tropical species in this group survival is typically limited to a few weeks or months. Actual storage of recalcitrant seed is therefore usually not realistic if the seed is sensitive to low temperatures. Storage of these recalcitrant species amounts to keeping them alive for short period until they are sown in the field.

2.1.2 Moisture Content

Moisture content is the most important factor affecting the longevity of cryopreserved seeds. Therefore it is crucial to determine the moisture content to get higher recovery in germination. This is reported by Krishnapillay *et al.* (1994) in *Pterocarpus indicus* seeds where the

moisture content between 14 - 16% only showed 26.0% of germination, compared to 86.0% germination if the moisture content is reduced to 8 - 10%.

Pritchard *et al.* (1995) pointed out the effect of moisture content on *Araucaria hunsteinii* seeds in relation to germination and storage. They found that seed longevity was decreased as mean moisture content was reduced from 45 to 30% of fresh weight basis and the time predicted for the loss of one probit of germination, fell from 5 years and 175 days to 20 days.

2.1.3 Viability Assessment

Besides moisture content and germination, another factor for classifying the seeds is viability. Viability means that a seed is capable of germinating and producing a normal seedling (Copeland and McDonald, 1995). In other words, viability denotes the degree to which a seed is alive, metabolically active and possesses enzymes capable of catalyzing metabolic reactions needed for germination and seedling growth. In this assessment, tetrazolium is utilized to recognize the viability of seeds. Tetrazolium is a biochemical compound (2,3,5-triphenyl tetrazolium chloride) that functions in estimating the viability, assessing vigour and diagnosing physiological problems in particular seeds or tissues. Tetrazolium is a water soluble powder, white or light yellow in colour. The solution with concentration of 0.1, 0.5 and 1.0% on a weight/volume (w/v) basis is prepared with distilled water and must be stored in a cool dark place.

The test relies on the action of tetrazolium molecule to react with hydrogen atoms that was released as a result of dehydrogenase enzymes activity in living tissue (Zhang *et al.*, 2001). This result in the formation of a water insoluble carmine-red pigment called formazon, where

the seeds will colour red if they are viable. Duration and temperature for staining in the tetrazolium test can be varied depends on types of seed, tetrazolium concentration and method used.

2.2 Cryopreservation

Cryopreservation is the process that involves storage and conservation of a living organism in a very low temperature. The specimen that is kept has the ability to continue living when it is cultivated again in a normal environment at 27°C (Towill, 1991). It involves limiting of chemical reaction and metabolic activity for a period of time (Pritchard, 1995) and makes possible the long term stable storage of plant tissues, organs and embryo (Frinkle *et al.*, 1982).

First report about freezing of plants in liquid nitrogen was in 1960 by Sakai (Marta, 2001) where twigs of mulberry (*Morus*), willow (*Salix*) and poplar (*Populus*) were cooled down from room temperature to super-low temperatures without damage. Cryopreservation offers interesting technical possibilities for the conservation of valuable germplasm compared to conventional preservation systems. The storage of living biological material at ultra-low temperature which normally occur at or near the temperature of liquid nitrogen (-196°C) have been studied over various types of plant germplasm including seed, pollen, dormant bud meristems, active shoot tips and active meristems derived from whole plants and cultured tissues.

The seed, as relatively small, dormant, and anhydrous organ, is one of the most convenient systems available for long-term storage of genetic information. Seed storage should be ideal to conserve a pool of genes in a base collection, especially for wild species in imminent

danger of loss and where clonal integrity is less critical. According to Pritchard (1995), low storage costs, combined with ease of seed distribution and regeneration of whole plants from genetically diverse material, offer distinct advantages for the storage and conservation of seeds compared with other types of plant tissues, such as meristems and pollen.

Cryopreservation of seeds had been done successfully and resulted in longer storage period. These were reported in *Pterocarpus indicus* by Krishnapillay *et al.*, (1994) and *Piper nigrum* (black pepper) by Chaudhury and Chandel (1994). Studies on these different seeds indicated that the reductions of moisture content by dehydration either using silica gel or laminar air flow hood were appropriate methods for cryostorage.

Currently, four types of cryopreservation namely dehydration, encapsulation, slow freezing and vitrification had been applied to various kinds of living organisms.

2.2.1 Dehydration

Dehydration technique is used to dehydrate the water content in seeds or meristems and had been applied to various species of plants in cryopreservation process. Hornung *et al.*, (2001) stated that dehydration technique for cryopreservation of plant species is based on the successive osmotic and evaporation dehydration of plant cells. Zhang *et al.*, (2001) also reported that desiccation by air drying is one of the effective method for avoiding intracellular freezing, provided that sufficient free water is present in the samples. Dehydration is done by desiccating seeds or meristems under the laminar air flow hood, by using silica gel or a cryoprotective method using the plasmolysis by a progressive concentration of sucrose.

Dussert *et al.* (1998) reported the effect of desiccation duration on moisture content and development of normal seedlings of *Coffea arabica, Coffea costatifructa, Coffea racemosa* and *Coffea sessiliflora* seeds using silica gel. For all species, seed moisture content decreased from 0.28 - 0.37 g H_2O gdw^{-1} to 0.11 - 0.14 g H_2O gdw^{-1} throughout the 16 hours desiccation period. Their study also reported that at lower moisture contents, the percentage of seeds which developed into normal seedlings was significantly higher for *C. arabica* (84% of initial percentage) than for the other three species (19 - 30%).

Krishnapillay *et al.* (1994) observed on desiccation of seeds and excised embryo of *Pterocarpus indicus* using silica gel and laminar air flow hood. Their findings showed that it is important to dry seeds to moisture content around 4 - 6% and 5% for embryos. The use of sucrose in dehydration technique has been done to somatic embryos of walnut (*Juglans regia*) and Eastern black walnut (*Juglans nigra*) (Boucard *et al.*, 1994). The somatic embryos were dehydrated slowly by subculturing with a progressive increase in concentration of sucrose. The result showed that the walnut somatic embryos were particularly tolerant to high plasmolysis by high concentration of sucrose.

2.3 Thawing

Thawing process is usually done rapidly by immersing the cryotubes containing the samples in a water bath thermostated at around 35 - 40°C. The aim is to avoid fusion during thawing of the ice microcrystals formed during freezing (Normah, 1995). However, the freshly thawed specimen is very vulnerable and must be handled with care (Withers, 1998). According to Towill (1991), thawing is accomplished by immersing vials containing the cells or tips into a

water bath (~35 - 40°C) for a few seconds with warming rates about 200 - 500°C/min depending on the volume of liquid in the tube.

However, slow thawing is necessary depending on plant samples with which ones works. It is successfully applied in gooseberry and currant meristem by immersing the cryotubes in a water bath at room temperatures for 15 minutes (Reed and Yu, 1995)

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

A. odoratissimus seeds
Tetrazolium solutions (0.1, 0.5 and 1.0%)
Grease
Silica gel
Sucrose solutions (0.2, 0.4, 0.6, 0.8 and 1.0 M)
Liquid nitrogen
Captan 50WP
70% ethanol
Distilled water
Forceps
Beakers
Moist riverine sand
Desiccators
Laminar flow hood
Plant growth chamber
Petri dishes
Trays
Oven
Aluminium plate
Air-tight bottle
Cryotubes

3.2 Seed Material

High quality and matured *A. odoratissimus* seeds were used and obtained from Lundu and Serian Districts. Seeds were cleaned by separation process to remove wrinkle, broken, damage seeds and other inert matters to obtain a high quality seed lot. The seeds were surface sterilized by soaking them into 70% ethanol for 5 minutes. Then, these seeds were rinsed three times with distilled water and air dried for 2 - 3 hours in the laminar flow to remove excess water. Seeds were dusted and mixed with Captan 50WP to protect them from fungi infection. Then seeds were kept into an air-tight container for used in the subsequent experiments.

3.3 Method

Standard tests such as moisture content, viability and germination were conducted in order to assess the quality of seeds as germplasm before and after treatments.

3.3.1 Moisture Content Test

Four replicates of 15 seeds per replicate each were used for evaluation of the moisture content. The seeds were placed and arranged on an aluminium plate and weighed to determine their wet weight. Then, the seeds were put in an oven at 60°C for 48 hours and after which they were removed and reweighed for dry weight (AOSA, 1985).

$$\text{Moisture Content (\%)} = \frac{(b) - (c)}{(b) - (a)} \times 100\%$$

where;

(a) = aluminium plate container

(b) = aluminium plate container + seeds weight before dried

(c) = aluminium plate container + seeds weight after dried

3.3.2 Seeds Viability (TZ) Test

There are numerous tests exist for determining seed viability. Viability (tetrazolium) test were used in this study. This method distinguishes between viable and dead seeds on the basis of their relative respiration rate in the hydrated state. Viability (TZ) test was done using solutions of 2,3,5-triphenyl tetrazoliumchloride (Zhang *et al.*, 2001). Three different concentrations of

tetrazolium were prepared which are 0.1, 0.5 and 1.0%. A preliminary test was conducted to obtain a suitable concentration and period for staining of seeds for 0, 1, 2, 3, 4, and 5 hours.

Four replicates of 50 seeds each were used for evaluation of suitable staining concentration and period. Before the seeds is stained, seed coat was removed either by using a sharp forceps or thumbnail. The seeds were counted as viable when they were stained completely red or with radicle and cotyledons prominently stained red (AOSA, 1970).

The seeds were placed in beakers and were put in an oven at 35°C for the above periods of staining. At the end of each period of staining, beakers were removed from the oven and stained seeds were rinsed several times in running tap water and then analyzed.

3.3.3 Standard Germination Test

To determine the germinability of seed, the standard germination test was conducted using moist riverine sand. Four replicates of 50 seeds each were used, where seeds were arranged on the sand medium. The seeds were left to germinate by incubating them in a plant growth chamber at a constant temperature of 29°C (Poehlman, 1991) with light source set alternately for 12 hours dark and 12 hours light. The percentage for seed germination were counted and recorded for every two days up to one week. Seed which produced a root (radicle) of approximately 2 mm or more in length was considered as germinated (ISTA, 1999).

3.3.4 Dehydration

Dehydration technique is used to dehydrate moisture content in seeds. This technique had been applied to various species of plants in cryopreservation process. Three dehydration

techniques were used and these were laminar air flow, silica gel and sucrose solution. Four replicates of 50 seeds each were used in each dehydration process whereby these seeds were obtained from the same seed lot for all the techniques mentioned above.

3.3.4.1 Laminar Air Flow Dehydration

Dehydration using air flow generated in laminar flow is a modification technique from Chaudhury and Chandel (1995). The seeds were placed on trays in a single layer to enhance the effectiveness of drying process. Dehydration of seeds was set at intervals of 0, 4, 8, 12, 16, 20 and 24 hours. After each period, seeds were evaluated for their moisture content, viability and germination. The best result recorded was selected and used in the subsequent experiments.

3.3.4.2 Silica Gel Dehydration

Silica gel was dried in an oven at 60°C for two days before it was used as dehydrating materials. Approximately 250 g of the silica gel was placed at the bottom of each desiccator and to minimize air from sipping in, grease was applied onto the lid of the desiccators. The seeds were put on a dry petri dish each and then were placed in desiccators to dehydrate for 0, 24, 48, 72 and 96 hours. After each period, moisture content, viability and germination test were conducted and the percentages were determined. The best result recorded was selected and used in the subsequent experiments.

3.3.4.3 Sucrose Solution Dehydration

Dehydration using different concentrations of sucrose was conducted using 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M solutions and intervals of dehydration were 0, 60, 120, 180 and 240 minutes.